



Comparison of the Sensititre YeastOne and CLSI M38-A2 Microdilution Methods in Determining the Activity of Amphotericin B, Itraconazole, Voriconazole, and Posaconazole against *Aspergillus* Species

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ABSTRACT This study compared the YeastOne and reference CLSI M38-A2 broth microdilution methods for antifungal susceptibility testing of *Aspergillus* species. The MICs of antifungal agents were determined for 100 *Aspergillus* isolates, including 54 *Aspergillus fumigatus* (24 TR₃₄/L98H isolates), 23 *A. flavus*, 13 *A. terreus*, and 10 *A. niger* isolates. The overall agreement (within 2 2-fold dilutions) between the two methods was 100%, 95%, 92%, and 90% for voriconazole, posaconazole, itraconazole, and amphotericin B, respectively. The voriconazole geometric mean (GM) MICs were nearly identical for all isolates using both methods, whereas the itraconazole and posaconazole GM MICs obtained using the YeastOne method were approximately 1 dilution lower than those obtained using the reference method. In contrast, the amphotericin B GM MIC obtained using the YeastOne method was 3.3-fold higher than that observed using the reference method. For the 24 *A. fumigatus* TR₃₄/L98H isolates assayed, the categorical agreement (classified according to the CLSI epidemiological cutoff values) was 100%, 87.5%, and 83.3% for itraconazole, voriconazole, and posaconazole, respectively. For four *A. niger* isolates, the itraconazole MICs were >8 µg/ml using the M38-A2 method due to trailing growth, whereas the corresponding itraconazole MICs obtained using the YeastOne method were all ≤0.25 µg/ml without trailing growth. These data suggest that the YeastOne method can be used as an alternative for azole susceptibility testing of *Aspergillus* species and for detecting the *A. fumigatus* TR₃₄/L98H isolates but that this method fails to detect *A. niger* isolates exhibiting trailing growth with itraconazole. Additionally, for isolates with azole MICs that approach or that are at susceptibility breakpoints or with high amphotericin B MICs detected using the YeastOne method, further MIC confirmation using the reference CLSI method is needed.

KEYWORDS amphotericin B, antifungal susceptibility, *Aspergillus*, azoles, CLSI M38-A2, Sensititre YeastOne, TR₃₄/L98H, trailing growth

The number of patients with invasive aspergillosis has increased over the years owing to a growing population of immunocompromised patients. The recommended therapies for aspergillosis primarily include voriconazole and isavuconazole, along with other mold-active azoles (itraconazole and posaconazole) and amphotericin B-based regimens as alternative therapies (1). However, multi-azole-resistant *Aspergillus fumigatus* strains harboring environmentally derived resistance mutations in the *cyp51A* gene (TR₃₄/L98H and TR₄₆/Y121F/T289A) have been increasingly reported worldwide over the past 2 decades (2). Isolates of *Aspergillus flavus* and *Aspergillus terreus* tend to exhibit reduced susceptibility to amphotericin B, and isolates with acquired azole

Received 10 May 2018 Returned for modification 14 June 2018 Accepted 30 July 2018

Accepted manuscript posted online 9 August 2018

Citation Wang H-C, Hsieh M-I, Choi P-C, Wu C-J. 2018. Comparison of the Sensititre YeastOne and CLSI M38-A2 microdilution methods in determining the activity of amphotericin B, itraconazole, voriconazole, and posaconazole against *Aspergillus* species. J Clin Microbiol 56:e00780-18. <https://doi.org/10.1128/JCM.00780-18>.

Editor David W. Warnock

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resistance have also been reported (1, 3, 4). Resistance to itraconazole is not uncommon among *Aspergillus niger* isolates, suggesting an intrinsic resistance to itraconazole in this species (3). Considering these intrinsic and acquired resistances to antifungal agents and the dismal outcome associated with resistance, performing antifungal susceptibility tests on clinically relevant *Aspergillus* isolates from patient groups or regions with known azole resistance is recommended (1). International expert opinion also suggests that voriconazole monotherapy be avoided as initial therapy for aspergillosis caused by *A. fumigatus* in regions with environmental azole resistance rates of $\geq 10\%$ (5).

A Clinical and Laboratory Standards Institute (CLSI) subcommittee has developed reproducible procedures for antifungal susceptibility testing of filamentous fungi using the broth microdilution method (document M38-A in 2002 and a revised document, M38-A2, in 2008) (6). However, the main disadvantage of the M38-A2 method is the need to prepare microdilution plates, which is time-consuming and impractical for routine use in clinical microbiology laboratories. Sensititre YeastOne is a commercial colorimetric microdilution assay consisting of prepared plates that are coated with dried serial 2-fold dilutions of antifungal agents. This test requires only the addition of medium containing the fungal inoculum, and MICs are determined on the basis of a color change of an oxidation-reduction indicator (alamarBlue). Sensititre YeastOne has been approved by the U.S. Food and Drug Administration (FDA) for *Candida* species but not for filamentous fungi (7).

Previous studies demonstrated that the MICs of itraconazole, posaconazole, and voriconazole obtained using the YeastOne method compared well with those obtained using the M38-A method for filamentous fungi, while the comparison results for amphotericin B MICs varied among studies (8–12). However, these surveys were primarily conducted before 2008, and azole-resistant *A. fumigatus* isolates with environmental resistance mechanisms have not yet been evaluated. Thus, the present study compared the YeastOne and reference CLSI M38-A2 methods for testing the susceptibility of common *Aspergillus* species, including *A. fumigatus* isolates with and without TR₃₄/L98H mutations, *A. flavus*, *A. terreus*, and *A. niger*, to amphotericin B, itraconazole, posaconazole, and voriconazole to investigate whether the Sensititre YeastOne method is suitable for detecting azole-resistant strains and for routine use in clinical laboratories.

MATERIALS AND METHODS

Aspergillus isolates. One hundred isolates were evaluated in this study, including 27 *A. fumigatus* (1 carried the TR₃₄/L98H mutations), 23 *A. flavus*, 13 *A. terreus*, and 8 *A. niger* clinical isolates from the National Cheng-Kung University Hospital isolated from 2011 to 2015; 4 *A. fumigatus* isolates (all carried the TR₃₄/L98H mutations) and 2 *A. niger* clinical isolates from our multicenter collection isolated during 2016 and 2017; and 23 *A. fumigatus* environmental isolates (7 carried the TR₃₄/L98H mutations and 12 carried the TR₃₄/L98H/S297T/F495I mutations; both sets of isolates are described as TR₃₄/L98H isolates in this study) recovered from 2014 to 2016 from Taiwan for use in our earlier work (13). *Aspergillus* species were identified by their morphological characteristics and sequence analyses of their internal transcribed spacer (ITS) region and calmodulin genes, and the presence of TR₃₄/L98H(S297T/F495I) mutations in *cyp51A* in 24 *A. fumigatus* isolates was confirmed as described previously (13). The *cyp51A* sequences of 10 *A. niger* isolates were determined using the PCR primers and conditions described by Hashimoto et al. and were compared with the *cyp51A* sequence of *A. niger* CBS 513.88 to detect the presence of any substitution(s) (14).

CLSI M38-A2 broth microdilution. Amphotericin B, itraconazole, posaconazole, and voriconazole powders were purchased from the Sigma-Aldrich Company (St. Louis, MO, USA) and used to prepare microdilution plates. Drug dilutions were prepared following the 2-fold drug dilution scheme described in document M38-A2 to yield serial 2-fold drug dilutions (from 0.03 to 16 $\mu\text{g/ml}$), which were twice as concentrated as the final concentrations (6). The microdilution plates were stored at -70°C until use.

Aspergillus conidial inoculum suspensions were prepared from well-sporulated cultures (typically 3 days old) grown on potato dextrose agar and adjusted spectrophotometrically to a turbidity that ranged from 0.4 to 0.7 McFarland standards at 530 nm. For the M38-A2 method, the suspension was then diluted with RPMI 1640 broth to twice the density needed for the final inoculum density (0.4×10^4 to 5×10^4 CFU/ml), as demonstrated by quantitative colony counts.

For testing, each microdilution well containing 100 μl of a solution with the 2 \times drug concentration was inoculated with 100 μl of the 2 \times inoculum suspension. The final volume and inoculum density in each well were 200 μl and 0.4×10^4 to 5×10^4 CFU/ml, respectively, and the final drug concentrations

were from 0.015 to 8 $\mu\text{g/ml}$. Growth (drug-free) and fungus-free controls were included. The microdilution plates were incubated at 35°C and examined for the MICs after 48 h. The MIC was the lowest drug concentration that prevented any discernible growth (100% inhibition).

YeastOne broth microdilution. Sensititre YeastOne panels (Trek Diagnostic Systems, Ltd., East Grinstead, United Kingdom) containing serial 2-fold dilutions of amphotericin B (0.12 to 8 $\mu\text{g/ml}$), itraconazole (0.015 to 16 $\mu\text{g/ml}$), posaconazole (0.008 to 8 $\mu\text{g/ml}$), and voriconazole (0.008 to 8 $\mu\text{g/ml}$) and the growth indicator (alarBlue) were used in this assay. The conidial suspensions were prepared as described for the M38-A2 method, and the inoculum suspensions were diluted with YeastOne broth according to the manufacturer's instructions to a final inoculum density of 0.4×10^4 to 5×10^4 CFU/ml, as demonstrated by quantitative colony counts.

For testing, the dried YeastOne panels were rehydrated with 100 μl of the working suspension in each well. The panels were covered with seal strips, incubated at 35°C, and read after 48 h. Fungal growth in the wells was indicated by a color change from blue (negative) to pink (positive), and the MIC was interpreted as the lowest drug concentration at which the growth indicator remained blue.

Candida parapsilosis ATCC 22019 and *A. fumigatus* ATCC MYA 3626 were used as quality control and reference strains, respectively, for susceptibility testing by both methods.

Reproducibility. To assess the reproducibility of both methods, the determination of MICs was repeated using both methods for isolates exhibiting MICs that differed by more than 1 2-fold dilution by the two methods and for isolates with an amphotericin B MIC of $>2 \mu\text{g/ml}$ by either method.

Data analysis. MIC ranges, $\text{MIC}_{50}/\text{MIC}_{90}$ values, and the geometric mean (GM) MICs were calculated using Microsoft Excel 2016 software for each species and method. The high off-scale MICs were converted to the next highest concentration, and the low off-scale MICs were left unchanged as the lowest tested concentrations. However, for isolates with an itraconazole MIC of $>16 \mu\text{g/ml}$ using the YeastOne method, the MIC was calculated as 16 $\mu\text{g/ml}$ to make it comparable to the MIC observed using the reference M38-A2 method (the upper MIC limit was set at 8 $\mu\text{g/ml}$). Discrepancies between MIC endpoints of no more than 2 2-fold dilutions were used to calculate the percentage of agreement between the two methods, as described by Martin-Mazuelos et al. (12). The non-wild-type MICs for *A. fumigatus* were classified according to the CLSI epidemiological cutoff values (ECVs), i.e., $>2 \mu\text{g/ml}$ for amphotericin B; $>1 \mu\text{g/ml}$ for itraconazole and voriconazole, as recommended by the CLSI M59 document (15); and $>0.25 \mu\text{g/ml}$ for posaconazole, as proposed by Pfaller et al. (16). A major error was defined as non-wild type according to the YeastOne method but as wild type according to the M38-A2 method, whereas a very major error was defined as wild type according to the YeastOne method but non-wild type according to the M38-A2 method.

This study was approved by the Institutional Review Boards at the National Health Research Institutes, Taiwan (no. EC1040502-E and EC1050307), and the National Cheng-Kung University Hospital (B-ER-101-342).

RESULTS AND DISCUSSION

MIC ranges, $\text{MIC}_{50}/\text{MIC}_{90}$ values, and GM MICs for 100 isolates evaluated by the YeastOne and M38-A2 methods and their agreement are shown in Table 1. The MICs for the quality control and reference strains for both methods were within the expected ranges. Overall, 75, 63, 66, and 51 isolates were tested twice with amphotericin B, itraconazole, posaconazole, and voriconazole, respectively. The reproducibility (within 1 2-fold dilution) of the methods for these drugs was excellent: 98.7%, 95.2%, 97.0%, and 96.1%, respectively, for the YeastOne method and 100%, 96.8%, 98.5%, and 98.0%, respectively, for the M38-A2 method.

The overall agreement (within 2 2-fold dilutions) between the two methods was the best for voriconazole (100%), followed by posaconazole (95%), itraconazole (92%), and amphotericin B (90%). Discrepancies were primarily due to the lower posaconazole and itraconazole MICs and higher amphotericin B MICs observed for the YeastOne method than for the M38-A2 method. For all isolates, the voriconazole GM MICs determined by the two methods were nearly identical (0.732 versus 0.758 $\mu\text{g/ml}$), whereas the itraconazole and posaconazole GM MICs obtained using the YeastOne method were approximately 1 2-fold dilution lower than those observed using the M38-A2 method (for itraconazole, 0.346 versus 0.688 $\mu\text{g/ml}$; for posaconazole, 0.096 versus 0.214 $\mu\text{g/ml}$). In contrast, the amphotericin B GM MIC observed in the YeastOne assay was 3.3-fold higher than that observed in the M38-A2 assay (1.959 versus 0.602 $\mu\text{g/ml}$). The high percentage of agreement ($>90\%$) for the itraconazole, voriconazole, and posaconazole MICs obtained using the two methods was consistent with the results from previous studies on *Aspergillus* spp. (8–12). Nevertheless, the comparison results for amphotericin B MICs differed among the studies. Our findings were close to those of a previous study that reported a 93.4% agreement between the two methods and a 1.6-fold higher GM MIC by the YeastOne method than by the M38-A method (12).

TABLE 1 MIC ranges, MIC₅₀/MIC₉₀ values, and GM MICs for *Aspergillus* species observed using the Sensititre YeastOne and reference CLSI M38-A2 methods and the agreement between the two methods^a

Antifungal agent, <i>Aspergillus</i> species (no. of isolates)	MIC (μg/ml)		No. (%) of isolates for which MICs determined by YeastOne differed from MICs determined by M38-A2 at the indicated dilution												
	Sensititre YeastOne														
	GM	50%/90%	Range	GM	50%/90%	Range	≥3	2	1	0	-1	-2	≤-3	Within ±1	Within ±2
Amphotericin B															
All (100)	1.959	2/4	1 to 4	0.602	0.5/1	0.12 to 2	10 (10)	49 (49)	39 (39)	2 (2)	0	0	0	41 (41)	90 (90)
<i>A. fumigatus</i> (all) (54)	1.828	2/2	1 to 4	0.526	0.5/1	0.25 to 1	4 (7.4)	36 (66.7)	13 (24.1)	1 (1.9)	0	0	0	14 (25.9)	50 (92.6)
<i>A. fumigatus</i> (WT) (30)	1.823	2/2	1 to 4	0.524	0.5/1	0.25 to 1	3 (10)	18 (60)	9 (30)	0	0	0	0	9 (30)	27 (90)
<i>A. fumigatus</i> (TR ₃₄) (24)	1.834	2/2	1 to 4	0.530	0.5/1	0.25 to 1	1 (4.2)	18 (75)	4 (16.7)	1 (4.2)	0	0	0	5 (20.8)	23 (95.8)
<i>A. flavus</i> (23)	2.470	2/4	2 to 4	1.095	1/2	0.5 to 2	0	5 (21.7)	17 (73.9)	1 (4.3)	0	0	0	18 (78.3)	23 (100)
<i>A. terreus</i> (13)	1.896	2/2	1 to 4	0.766	1/1	0.5 to 2	0	4 (30.8)	9 (69.2)	0	0	0	0	9 (69.2)	13 (100)
<i>A. niger</i> (10)	1.741	2/2	1 to 2	0.230	0.25/0.5	0.12 to 0.5	6 (60)	4 (40)	0	0	0	0	0	0 (0)	4 (40)
Itraconazole															
All (100)	0.346	0.12/>16	<0.015 to >16	0.688	0.25/>8	0.015 to >8	0	0	1 (1)	44 (44)	32 (32)	15 (15)	8 (8)	77 (77)	92 (92)
<i>A. fumigatus</i> (all) (54)	0.906	0.12/>16	<0.015 to >16	1.644	0.5/>8	0.06 to >8	0	0	0	26 (48.1)	15 (27.8)	9 (16.7)	4 (7.4)	41 (75.9)	50 (92.6)
<i>A. fumigatus</i> (WT) (30)	0.091	0.12/0.12	<0.015 to 0.25	0.266	0.25/0.5	0.06 to 0.5	0	0	0	2 (6.7)	15 (50)	9 (30)	4 (13.3)	17 (56.7)	26 (86.7)
<i>A. fumigatus</i> (TR ₃₄) (24)	>16	>16	>16	>8	>8	>8	0	0	0	24 (100)	0	0	0	24 (100)	24 (100)
<i>A. flavus</i> (23)	0.120	0.12/0.12	0.06 to 0.25	0.175	0.12/0.5	0.12 to 0.5	0	0	1 (4.3)	12 (52.2)	7 (30.4)	3 (13.0)	0	20 (87.0)	23 (100)
<i>A. terreus</i> (13)	0.067	0.06/0.12	0.06 to 0.12	0.114	0.12/0.12	0.06 to 0.25	0	0	0	4 (30.8)	8 (61.5)	1 (7.7)	0	12 (92.3)	13 (100)
<i>A. niger</i> (10)	0.186	0.25/0.25	0.12 to 0.25	1.510	0.5/>8	0.12 to >8	0	0	0	2 (20)	2 (20)	2 (20)	4 (40)	4 (40)	6 (60)
Posaconazole															
All (100)	0.096	0.06/0.5	<0.008 to 1	0.214	0.12/1	0.03 to 2	0	0	1 (1)	22 (22)	43 (43)	29 (29)	5 (5)	66 (66)	95 (95)
<i>A. fumigatus</i> (all) (54)	0.112	0.06/0.5	<0.008 to 1	0.321	0.25/2	0.03 to 2	0	0	0	3 (5.6)	24 (44.4)	23 (42.6)	4 (7.4)	27 (50)	50 (92.6)
<i>A. fumigatus</i> (WT) (30)	0.035	0.03/0.06	<0.008 to 0.12	0.113	0.12/0.25	0.03 to 0.25	0	0	0	2 (6.7)	10 (33.3)	14 (46.7)	4 (13.3)	12 (40.0)	26 (86.7)
<i>A. fumigatus</i> (TR ₃₄) (24)	0.472	0.5/0.5	0.25 to 1	1.189	1/2	0.5 to 2	0	0	0	1 (4.2)	14 (58.3)	9 (37.5)	0	15 (62.5)	24 (100)
<i>A. flavus</i> (23)	0.110	0.12/0.12	0.06 to 0.12	0.146	0.12/0.25	0.06 -0.25	0	0	0	14 (60.9)	9 (39.1)	0	0	23 (100)	23 (100)
<i>A. terreus</i> (13)	0.048	0.06/0.06	0.03 to 0.12	0.083	0.06/0.12	0.06 to 0.12	0	0	1 (7.7)	4 (30.8)	5 (38.5)	3 (23.1)	0	10 (76.9)	13 (100)
<i>A. niger</i> (10)	0.074	0.06/0.12	0.03 to 0.25	0.199	0.12/0.5	0.06 to 0.5	0	0	0	1 (10)	5 (50)	3 (30)	1 (10)	6 (60)	9 (90)
Voriconazole															
All (100)	0.732	0.5/4	0.12 to 8	0.758	0.5/4	0.12 to 4	0	0	8 (8)	79 (79)	13 (13)	0	0	100 (100)	100 (100)
<i>A. fumigatus</i> (all) (54)	0.913	0.5/4	0.12 to 8	0.999	0.5/4	0.12 to 4	0	0	2 (3.7)	43 (79.6)	9 (16.7)	0	0	54 (100)	54 (100)
<i>A. fumigatus</i> (WT) (30)	0.425	0.5/0.5	0.12 to 0.5	0.455	0.5/0.5	0.12 to 1	0	0	1 (3.3)	25 (83.3)	4 (13.3)	0	0	30 (100)	30 (100)
<i>A. fumigatus</i> (TR ₃₄) (24)	2.378	2/4	1 to 8	2.670	4/4	1 to 4	0	0	1 (4.2)	18 (75)	5 (20.8)	0	0	24 (100)	24 (100)
<i>A. flavus</i> (23)	0.599	0.5/1	0.5 to 1	0.547	0.5/1	0.25 to 1	0	0	3 (13.0)	20 (87.0)	0	0	0	23 (100)	23 (100)
<i>A. terreus</i> (13)	0.474	0.5/0.5	0.25 to 1	0.474	0.5/0.5	0.25 to 0.5	0	0	2 (15.4)	9 (69.2)	2 (15.4)	0	0	13 (100)	13 (100)
<i>A. niger</i> (10)	0.616	0.5/1	0.25 to 1	0.660	0.5/1	0.25 to 2	0	0	1 (10)	7 (70)	2 (20)	0	0	10 (100)	10 (100)

^aAbbreviations: GM, geometric mean; TR₃₄, TR₃₄/L98H/S297T/F495I mutations in *cyp51A*; WT, the wild-type *cyp51A* or *cyp51A* nucleotide polymorphism(s) not associated with azole resistance.

TABLE 2 Amphotericin B MICs determined using the Sensititre YeastOne and CLSI M38-A2 methods for *Aspergillus* species

<i>Aspergillus</i> species, MIC (μ g/ml) by YeastOne	No. (%) of isolates with the following MIC (μ g/ml) by CLSI M38-A2 method:					
	0.12	0.25	0.5	1	2	All
<i>A. fumigatus</i> (n = 54)						
1	0	4 (7.4)	7 (13.0)	1 (1.9)	0	12 (22.2)
2	0	1 (1.9)	30 (55.6)	6 (11.1)	0	37 (68.5)
4	0	0	3 (5.6)	2 (3.7)	0	5 (9.3)
All	0	5 (9.3)	40 (74.1)	9 (16.7)	0	54 (100)
<i>A. flavus</i> (n = 23)						
1	0	0	0	0	0	0
2	0	0	2 (8.7)	13 (56.5)	1 (4.3)	16 (69.6)
4	0	0	0	3 (13.0)	4 (17.4)	7 (30.4)
All	0	0	2 (8.7)	16 (69.6)	5 (21.7)	23 (100)
<i>A. terreus</i> (n = 13)						
1	0	0	2 (15.4)	0	0	2 (15.4)
2	0	0	4 (30.8)	6 (46.2)	0	10 (76.9)
4	0	0	0	0	1 (7.7)	1 (7.7)
All	0	0	6 (46.2)	6 (46.2)	1 (7.7)	13 (100)
<i>A. niger</i> (n = 10)						
1	1 (10)	1 (10)	0	0	0	2 (20)
2	3 (30)	2 (20)	3 (30)	0	0	8 (80)
4	0	0	0	0	0	0
All	4 (40)	3 (30)	3 (30)	0	0	10 (100)

However, our findings differed from those of a previous survey showing that lower MICs were obtained with the YeastOne method than with the M38-A method and that the results of the two methods had a low agreement (31.5%) (10).

The amphotericin B GM MICs for *A. fumigatus*, *A. flavus*, *A. terreus*, and *A. niger* (0.526, 1.095, 0.766, and 0.230 μ g/ml, respectively) and MIC₅₀ values for the same four species (0.5, 1, 1, and 0.25 μ g/ml, respectively) determined using the M38-A2 method in this study were in line with previously reported amphotericin B MICs among *Aspergillus* spp., in which *A. flavus* and *A. terreus* exhibited higher MICs than *A. fumigatus* and *A. niger* exhibited the lowest MIC among the four species (4, 9). For the above-mentioned species, Espinel-Ingroff and colleagues reported GM MICs of 0.67, 0.96, 1.8, and 0.3 μ g/ml, respectively, and MIC₅₀ values of 0.5, 1, 2, and 0.5 μ g/ml, respectively, in 2006 and 2011 (4, 9). Furthermore, all isolates tested in this study were inhibited by 2 μ g/ml amphotericin B (by the M38-A2 method), similar to the findings of Espinel-Ingroff et al. (4) that 99.7% (3,977/3,988) of *A. fumigatus* isolates and 97.4% (772/793) of *A. flavus* isolates had MICs of ≤ 2 μ g/ml and those of Castanheira et al. (17) that all 391 *A. fumigatus* isolates assayed had MICs of ≤ 2 μ g/ml. On the other hand, YeastOne yielded higher amphotericin B MICs, as the MIC₅₀ values for all four species were 2 μ g/ml, with 9.3% (5/54) of *A. fumigatus* isolates and 30.4% (7/23) of *A. flavus* isolates exhibiting MICs of > 2 μ g/ml (Table 2), demonstrating a major error rate of 9.3% for *A. fumigatus* using this method. The high amphotericin B MICs obtained using the YeastOne method have been observed elsewhere. For example, Dunne et al. reported that the use of YeastOne resulted in an unexpectedly high percentage of *A. fumigatus* respiratory isolates from patients with cystic fibrosis with high amphotericin B MICs (2 μ g/ml) (21.4%, 12/56) (18), and Li et al. reported that 9.3% (10/108) of *A. fumigatus* isolates and 41.9% (39/93) of *A. flavus* isolates had MICs of > 2 μ g/ml using the YeastOne method (19). Insufficient categorical agreement (72.6%) between the YeastOne and CLSI M27-A3 methods for amphotericin B MICs has also been reported for *Candida* and *Cryptococcus*; in these cases, the YeastOne MICs were higher than the reference MICs, resulting in a major error rate of 26.5% (resistant according to the YeastOne assay results but susceptible according to the M27-A3 assay results) (20). Collectively, the YeastOne method ap-

TABLE 3 Categorical agreement between the YeastOne and CLSI M38-A2 assay results for 24 *A. fumigatus* TR₃₄/L98H isolates based on the CLSI ECV proposed by CLSI M59 and Pfaller et al.^a

Antifungal agent	CLSI ECV ($\mu\text{g/ml}$) for non-wild type	Method	No. (%) of isolates categorized on the basis of the ECV		% error		
			Wild type	Non-wild type	CA	VME	ME
Itraconazole	>1	YeastOne	0 (0)	24 (100)	100	0	0
		M38-A2	0 (0)	24 (100)			
Posaconazole	>0.25	YeastOne	4 (16.7)	20 (83.3)	83.3	16.7	0
		M38-A2	0 (0)	24 (100)			
Voriconazole	>1	YeastOne	6 (25)	18 (75)	87.5	12.5	0
		M38-A2	3 (12.5)	21 (87.5)			

^aThe cutoff values proposed by CLSI M59 (15) and Pfaller et al. (16) have been described previously. Abbreviations: ECV, epidemiological cutoff value; CA, categorical agreement; ME, major error; VME, very major error.

peared to yield higher amphotericin B MICs than the reference CLSI method. However, the underlying contributing factors remain unknown, warranting further investigation.

Of the 24 *A. fumigatus* TR₃₄/L98H isolates assayed, non-wild-type MICs for itraconazole, posaconazole, and voriconazole were observed in 100%, 100%, and 87.5% of the isolates, respectively, using the M38-A2 method and in 100%, 83.3%, and 75% of the isolates, respectively, using the YeastOne method (Table 3). The results obtained using the M38-A2 method agreed with previous reports showing that TR₃₄/L98H isolates were characterized by a high itraconazole MIC (>8 $\mu\text{g/ml}$), cross-resistance to posaconazole, and variable susceptibility to voriconazole (21, 22). Overall, the itraconazole and voriconazole MICs determined using the YeastOne and M38-A2 methods correlated well in terms of the observed GM MICs, MIC ranges, and the high level of agreement (within 1 2-fold dilution, 100% for itraconazole and voriconazole) (Table 1). The categorical agreement was 100%, 87.5%, and 83.3% for itraconazole, voriconazole, and posaconazole, respectively (Table 3). The very major error rates noted for voriconazole (12.5%) and posaconazole (16.7%) were explained by 3 isolates with lower voriconazole MICs by the YeastOne method than by the reference method and 4 isolates with lower posaconazole MICs by the YeastOne method than by the reference method (1 $\mu\text{g/ml}$ versus 2 $\mu\text{g/ml}$, respectively, for voriconazole and 0.25 $\mu\text{g/ml}$ versus 1 $\mu\text{g/ml}$, respectively, for posaconazole). These observations highlight the importance of further confirmation of the MIC using the reference CLSI method for isolates with azole MICs that approach or that are at susceptibility breakpoints using the YeastOne method. Although major errors were noted, the high categorical agreement of itraconazole values indicated that the YeastOne method could detect itraconazole-resistant *A. fumigatus* TR₃₄/L98H isolates. Nevertheless, our study has two limitations regarding azole susceptibility testing. First, we did not include *A. fumigatus* isolates with TR₄₆/Y121F/T289A mutations, which are known for their high-level resistance to voriconazole (MIC > 16 $\mu\text{g/ml}$) and that have yet to be identified in Taiwan (13, 21). However, on the basis of the high level of agreement in the voriconazole MICs determined using the two methods, the YeastOne method could, theoretically, also be used to detect voriconazole-resistant strains with such mutations. Second, the performance of the YeastOne method in determining isavuconazole MICs is unclear since this agent is not included in the current YeastOne panel.

Using the M38-A2 method, four *A. niger* strains (F1872, 7987, C01-004, and C01-014) were observed to exhibit trailing growth with microscopically aberrant small, rounded, compact hyphal forms in wells with itraconazole concentrations ranging from 0.5 to 8 $\mu\text{g/ml}$. In contrast, normal hyphal growth was observed in the growth control and in wells with itraconazole concentrations of ≤ 0.25 $\mu\text{g/ml}$. Thus, the itraconazole MICs were read as >8 $\mu\text{g/ml}$ (Fig. 1), whereas all four strains remained susceptible to posaconazole and voriconazole, with MICs of ≤ 0.5 $\mu\text{g/ml}$ and ≤ 2 $\mu\text{g/ml}$, respectively, and lacked trailing growth. On the other hand, the corresponding itraconazole MICs determined using the YeastOne method were all ≤ 0.25 $\mu\text{g/ml}$ (see Fig. S1 in the supplemental material), and microscopically trailing growth was not observed. These

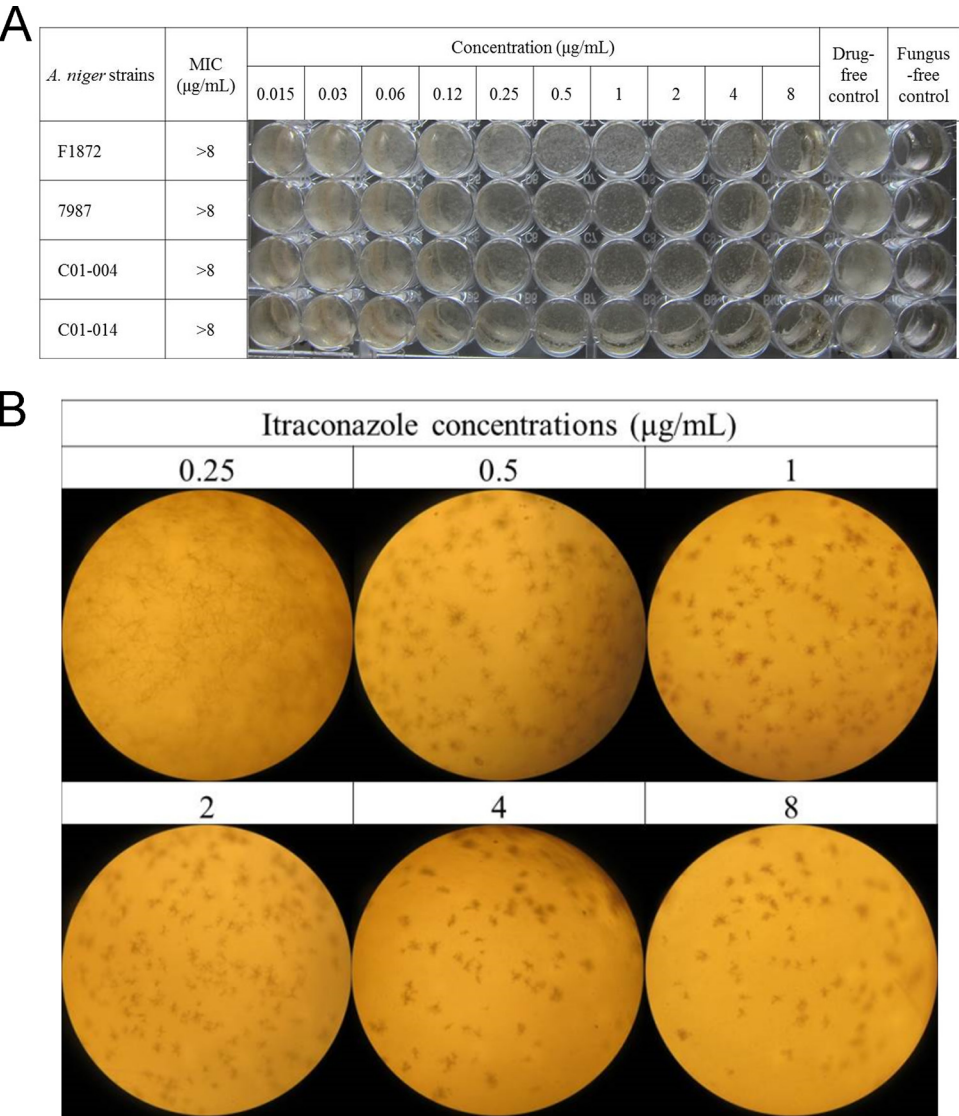


FIG 1 Determination of MICs of itraconazole against four *Aspergillus niger* strains using the CLSI M38-A2 method. In wells with itraconazole concentrations ranging from 0.5 to 8 μg/ml, trailing growth (A) and microscopically aberrant growth (small, rounded, compact hyphal forms, strain C01-014) (B) were observed. In contrast, normal hyphal growth occurred in the wells with an itraconazole concentration of 0.25 μg/ml.

four strains, each picked up from a single colony from a subculture plate, were tested with both methods thrice, and the results were reproducible. Of note, *cyp51A* sequence analyses revealed that strain C01-004 had N164V and G509E substitutions and strain C01-014 had a H382R substitution; these substitutions were not present in susceptible isolates (Table S1). The G509E substitution (orthologous to the G509E substitution in *A. niger*) could also be detected in two itraconazole-susceptible *Aspergillus welwitschiae* isolates and is therefore probably not associated with itraconazole resistance (14). The H382R substitution has been reported in an itraconazole-resistant *A. niger* strain (MIC = 8 μg/ml), whereas the N164V substitution has not yet been reported (14), and the association of both substitutions with itraconazole resistance needs further evaluation. Strain F1872 was obtained from the sputum of a patient with ventilator-associated pneumonia to whom an antimold agent was not given in time due to rapid mortality. Strain 7987 was obtained from the sputum of an elderly patient with chronic myeloid leukemia who was treated with voriconazole for possible pulmonary aspergillosis but who eventually died of uncontrolled leukemia and progressive bacterial and fungal

pneumonia. Strains C01-004 and C01-014 were collected from respiratory and inner ear samples, respectively, and their clinical data were not available. Until now, trailing growth in itraconazole-containing wells in the M38-A2 procedure has rarely been reported for *A. niger*, and differences in itraconazole MIC results from the M38-A2 and YeastOne methods due to the presence and absence of trailing growth, respectively, have also not been described. Although high itraconazole MICs ($>8 \mu\text{g/ml}$) are associated with clinical resistance (23), the clinical impact of trailing growth causing an elevation of itraconazole MICs is unclear due to the rarity of related reports. Further evaluations with more isolates and cases might be needed to determine the correlation between trailing growth and the clinical outcome.

In conclusion, our data suggest that the YeastOne method maybe a suitable alternative method for assessing the susceptibility of common *Aspergillus* species to itraconazole, posaconazole, and voriconazole and for detecting azole-resistant *A. fumigatus* TR₃₄/L98H isolates in clinical laboratories. However, the use of this method may result in lower azole MICs and a failure to detect *A. niger* isolates that exhibit trailing growth with itraconazole using the M38-A2 method. Additionally, the YeastOne method tended to yield higher amphotericin B MICs. Thus, for isolates with azole MICs that approach or that are at the susceptibility breakpoints or with high amphotericin B MICs determined using the YeastOne method, further MIC confirmation using the reference CLSI method is needed.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JCM.00780-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

ACKNOWLEDGMENTS

We gratefully thank Pei-Hsin Chou for her laboratory assistance and the Changhua Christian Hospital, Chi Mei Medical Center (Liouying), Kaohsiung Veterans General Hospital, National Cheng-Kung University Hospital, and Taichung Veterans General Hospital for sharing *Aspergillus* clinical isolates.

This study was supported by grants from the National Health Research Institutes, Taiwan (IV-104-PP-09, IV-105-PP-08, and IV-106-PP-08), and from the Ministry of Science and Technology, Taiwan (MOST 105-2628-B-400-004-MY2).

We have no conflicts of interest to declare.

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